Feeding inhibition tests as a tool for seston quality evaluation in lentic ecosystems: salinization impact

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

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Dissertação submetida à Faculdade de Ciências da Universidade do Porto para a obtenção do grau de Mestre em Ecologia e Ambiente, da responsabilidade do Departamento de Biologia.

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“Shoot for the moon. Even if you miss, you’ll land among the stars.”

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Resumo

As alterações ambientais nos ecossistemas aquáticos provocam alterações significativas nos diferentes níveis tróficos. A salinidade em ecossistemas dulçaquícolas lênticos, como consequência das alterações climáticas, é um assunto de preocupação da comunidade científica, especialmente nos ecossistemas localizados em zonas costeiras. Estas alterações afetam a qualidade da água e a composição e diversidade das comunidades aquáticas (fitoplâncton, zooplâncton e ictiofauna). Os objetivos deste estudo foram: i) aferir a qualidade do *seston* de dois ecossistemas dulçaquícolas lênticos sujeitos a pressões antrópicas distintas (albufeira de Crestuma e lagoa da Vela) em dois períodos distintos (final de verão – simulando *worst case scenario*; primavera – *best case scenario*) e ii) avaliar o efeito da salinidade na *performance* alimentar de *Daphnia magna* (organismo padrão em ecotoxicologia aquática) e *Daphnia longispina* (isolada dos dois ecossistemas naturais em estudo – *D. longispina* C (Crestuma) e *D. longispina* V (Vela)). Testes de inibição alimentar foram a metodologia utilizada para esta avaliação. Para cumprir o primeiro objetivo foi utilizada água recolhida nos dois locais do estudo e os tratamentos analisados foram com a água antes e após filtração. Efetuou-se a análise dos parâmetros físicos e químicos das amostras de água recolhidas, após as quais se verificou que apenas a água de Crestuma, no verão, possuía boa qualidade, e as restantes amostragens classificavam as massas de água como de má qualidade. Porém, os resultados obtidos nos testes de inibição alimentar, indicam que a qualidade nutricional do *seston* presente na lagoa da Vela é superior ao recolhido na albufeira de Crestuma. Regra geral, os organismos expostos à água filtrada da lagoa da Vela revelaram inibição significativa da taxa de alimentação. No entanto, quando *Daphnia* spp. foi exposta à água não filtrada deste local, registou-se um aumento significativo na taxa de alimentação dos organismos. Relativamente aos ensaios com a água da albufeira de Crestuma foi observada diminuição na taxa de alimentação de *Daphnia* spp. em ambos os tratamentos analisados. No tocante aos ensaios da avaliação do efeito da salinidade na taxa de filtração de *Daphnia* spp., observou-se uma redução significativa da taxa de alimentação para ambas as espécies. O NOEC observado para as espécies estudadas foi de 0.7 g/L de NaCl, enquanto que o LOEC foi distinto entre as espécies estudadas (*D. magna* – 1.0 g/L e *D. longispina* – 0.8 g/L). Este trabalho permitiu verificar que os ensaios de inibição alimentar não foram suficientemente sensíveis para a avaliação da qualidade de águas naturais. No entanto, são economicamente viáveis e possuem sensibilidade para serem utilizados na avaliação dos efeitos de NaCl em *Daphnia* spp..

Palavras-chave: *Daphnia* spp., qualidade da água, ecossistemas lênticos, inibição alimentar, salinidade, qualidade do seston

Abstract

Environmental disturbance on freshwater ecosystems significantly impacts all levels of the trophic web. Salinity in lentic freshwater ecosystems, as a consequence of climatic changes, is a matter of great concern to the scientific community, especially on those situated at coastal zones. These alterations affect water quality, the composition and diversity of the aquatic communities' (phytoplankton, zooplankton, and ictiofauna). This study aims to: i) assess seston quality of two lentic freshwater ecosystems subjects to different anthropic pressures (Crestuma reservoir and lake Vela) in two distinct seasons (end of summer – simulated a worst case scenario; and spring – addressing a best case scenario), and ii) evaluate the effects of increasing salinity on food performance of *Daphnia magna* (standard species in aquatic ecotoxicology) and *Daphnia longispina* (isolated from the two natural ecosystems studied – *D. longispina* C from Crestuma and *D.longispina* V from Vela). Feeding inhibition tests was the methodology used to conduct this evaluation. To accomplish the first objective, water samples from both study sites were used and treatments consisted of filtered and unfiltered water samples. A chemical and physical water analysis was performed with natural water and it was verified that only summer samples from Crestuma presented a good water quality, while the remaining natural waters were classified as poor quality. However, the results from the feeding inhibition tests show that seston from lake Vela presents more nutritional quality than seston collected in Crestuma reservoir. In general, the organisms exposed to filtered water from lake Vela revealed a significant inhibition of the feeding rate. On the other hand, when *Daphnia* spp. was exposed to unfiltered water from this site, there was a significant increase in the feeding rate of those organisms. Regarding the assays with Crestuma water samples, a lower feeding rate in both treatments was observed in *Daphnia* spp.. Concerning assay to evaluate the salinity effect on *Daphnia* spp. a significant reduction in the filtration rate was observed for the both species tested. The NOEC value observed for the tested species was of 0.7 g/L NaCl, whilst the LOEC was distinct between species (*D. magna* – 1.0 g/L and *D. longispina* – 0.8 g/L). This work allowed verified that feeding inhibition tests are not sensitive enough for natural water quality evaluation. However, these tests are economically viable and have high sensitivity to be used in order to evaluate NaCl effects on *Daphnia* spp..

Keywords: *Daphnia* spp., water quality, lentic ecosystems, feeding inhibition, salinity, seston quality

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List of Abbreviations

P – Phosphorous

N – Nitrates

WFD – Water Frame Directive

APHA – American Public Health Association

ASTM - American Society for Testing and Materials

OECD - Organisation for Economic Co-operation and Development

EC – Lethal Concentration

LC – Lethal Dose

NOEC – No Observed Effect Concentration

LOEC – Lowest Observed Effect Concentration

INAG – Instituto Nacional da Água

GEP – Good Ecological Potential

SNIRH – Sistema Nacional de Informação de Recursos Hídricos

IPMA - Instituto Português do Mar e da Atmosfera

1. Introduction

Lentic freshwater ecosystems are water bodies that can provide habitat for several groups of organisms. and correspond to an essential natural water source. These ecosystems present diversified characteristics from temporary ponds to permanent water bodies (Hoverman & Johnson, 2012) such as lakes, ponds, and artificial modified water bodies - reservoirs. Aquatic ecosystems biodiversity is changing across the globe (Sala et al., 2000; Dudgeon et al., 2006) as a response to different anthropogenic threats and geologic characteristics. Namely, freshwater ecosystems are especially vulnerable since they are intensively explored and subjects to human-induced impacts (e.g. industrial chemicals, dams for energy production). Several studies already demonstrated that the occurrence of natural or anthropogenic disturbance can alter the ecosystems microhabitat characteristics and, consequently, affect the biota communities and the trophic web (Abrantes et al., 2006; Figueiredo et al., 2006; Kagalou et al., 2006; Angeler & Moreno, 2007; Yvon-Durocher et al., 2011; Edwards et al., 2016; Nõges et al., 2016; Hintz et al., 2017). On lentic freshwater ecosystems, the habitat characteristics depend on the range variation of biotic and abiotic factors. These parameters are responsible for the plankton community fluctuation and distribution (Antunes et al., 2003; Figueiredo et al., 2006; Choi et al., 2014). The occurrence of such modifications on these communities can cause significant impacts on the entire ecosystem and give rise to processes like eutrophication.

Eutrophication process is a worldwide environmental problem (Withers & Haygarth, 2007; Nørring & Jørgensen, 2009; Cruz et al., 2015) mainly caused by human activities, such agriculture or industry (Ansari et al., 2010). This process occurs as a consequence of excessive nutrients input, especially phosphorous (P) and nitrates (N), from diverse anthropogenic sources (e.g. industrial discharges, sewage, agriculture runoffs). The significant increase of these nutrients concentration may induce alterations in the structure (abnormal growth of primary producers - phytoplankton) and functioning of the ecosystems, that cause significant impacts in water quality (Ansari et al., 2010). Furthermore, in eutrophic ecosystems, the primary consumers (zooplankton population) become unable to filter and control the significant increase of phytoplankton with a degradation of water quality as the last consequence (von Ruckert & Giani, 2008; Gamito et al., 2017).

Zooplankton community plays an important role in freshwater ecosystems since it includes organisms with high capacity of modifying the structure of planktonic food webs, due to their predatory and grazing behavior. Zooplanktonic primary consumers, such as Cladocerans, can filter particles from seston, including bacteria and algae

(Leonard & Paerl, 2005; Marinho et al., 2018). Moreover, various studies had already verified the key role of this organisms in controlling the growth of phytoplankton communities and cyanobacteria blooms (Lampert et al., 1986; Christoffersen et al., 1993; Tan et al., 2004; Muylaert et al., 2006; Ger et al., 2014; George et al., 2015). On the other hand, these organisms have a limited food resource selection capacity and, consequently, the quality and quantity of food present in the seston is determinant for the performance of their life history (Ahlgren et al., 1990; Müller-Navarra & Lampert, 1996; Hülsmann, 2001; Müller-Solger et al., 2002; Marinho et al., 2018). It is known that the concentration and composition of the phytoplankton community are essential to the zooplanktonic community since different phytoplankton species show different nutritional quality (von Ruckert & Giani, 2008; Choi et al., 2014). However, abiotic factors, such as temperature, pH, nutrients, and salinity are also important factors that influence the grazing and survival of phytoplankton and zooplankton species (Arnott & Vanni, 1993; Elser et al., 2001; Gonçalves et al., 2007; George et al., 2015; Loureiro et al., 2015).

In the last decades, the salinity (abiotic stress) increase in freshwater ecosystems, and this situation has been an issue of rising concern in the scientific community (Berzas, 2000; Nielsen et al., 2003; Kaushal et al., 2005, 2018; Herbert et al., 2015; Canedo-Arguelles et al., 2016). Namely, freshwater ecosystems situated at coastal zones and subjects to different pressures of climatic changes (increase salinity and temperature) (Gonçalves et al., 2007; Venâncio et al., 2018). There are several causes of salinization on coastal freshwater ecosystems such as decrease on precipitation levels, and sea level rise, both consequences of global climate changes (Schallenberg et al., 2003; Herbert et al., 2015; Jeppesen et al., 2015). These alterations affect the water quality and, consequently, the plankton communities, which must adapt to saline stress in order to survive (Gonçalves et al., 2007). Several authors have already reported a significant loss of biodiversity in freshwater ecosystems as a consequence of salinity increase (Green & Mengestou, 1991; Ramdani et al., 2001; Schallenberg et al., 2003; Jeppesen et al., 2015).

Cladocera is the most important primary group of consumers in lentic ecosystems, and has been widely used to evaluate the impact of environmental changes due to their key position in the trophic web and sensibility at different stressors (Zhang et al. 2010; Jeppesen et al. 2011; Leitão et al. 2013; Loureiro et al. 2015; Lari et al. 2017; Venâncio et al. 2018). Aladin (1991) and Bezirci et al. (2012) verified that cladocera organisms presented a high sensitivity to osmotic stress and therefore an ideal bio-indicator of saline stress. Among cladocera, *Daphnia* species are already described as tolerant to salinity (Gonçalves et al., 2007). *Daphnia magna* is a well known standard laboratory organism, and there are various studies on the effect of salinity increase in this species. Namely, a significant decrease on growth, survival and life cycle parameters of this species was already demonstrated by several authors (Arnér & Koivisto, 1993; Gonçalves et al., 2007; Martínez-Jerónimo & Martínez-Jerónimo, 2007; Ghazy et al., 2009). Nevertheless, there is still little information on how the salinity affects others parameters in zooplankton species, namely in feeding rates.

Feeding inhibition tests have been used to assess effects of chemical compounds, pesticides, metal oxides, and cyanotoxins for example, on food performance of *Daphnia* spp. (McWilliam & Baird, 2002; Barata et al., 2007; Loureiro et al., 2010; Freitas et al., 2014; Lopes et al., 2014). Barata et al. (2008) have considered this tests as cost-effective and sensitive comparing to standardized *D. magna* acute and chronic tests. Therefore, these tests can potentially be used as an important tool to assess the seston quality of freshwater ecosystems or evaluate the effects of different stresses (e.g. salinity).

Aims

There is still a lack of knowledge on the effects of salinity on food performance of freshwater organisms. Therefore, this study defined two main objectives:

- to assess the seston quality of two lentic freshwater ecosystems subjected to different anthropic pressures, Crestuma reservoir and lake Vela, in two distinct periods (*end of summer – worst case scenario and spring – best case scenario*).
- to evaluate the effects of salinity in the feeding rate of *Daphnia* spp..

To achieve both objectives, feeding inhibition tests were performed with two *Daphnia* species: *Daphnia magna*, standard organisms commonly used on ecotoxicological tests, and *Daphnia longispina,* an autochtonous species isolated from two Portuguese freshwater ecosystems (Crestuma reservoir and lake Vela).

2. Materials and Methods

2.1 Natural lentic ecosystems

Two lentic freshwater ecosystems, subjects to different anthropic pressures, were chosen to assess the seston quality and to the sampling of *D. longispina* populations needed to perform the assays described below.

2.1.1 Crestuma-Lever reservoir

Crestuma-Lever reservoir (41º4'38.071"N, -8º28'20.406"W) belongs to the Douro river hydrographic basin and it is located in Vila Nova de Gaia and Gondomar municipalities (Porto district). In the Portuguese territory, this river is sectioned by 10 dams originating artificial reservoirs (Figure 1). Crestuma reservoir (constructed in 1985) is situated on the final stretch of the Douro river, 22 km from the sea. Consequently, any alteration throughout the hydrographic basin will affect the water quality of the reservoir. Since its formation, it is considered an artificial mesotrophic water mass (POACL, 2004). Crestuma-Lever reservoir is classified with multiple uses (Regulatory Decree 2/88 of January 20) and, at this moment, the main are consumption, irrigation, recreation activities and wastewater discharge.

Fig. 1 - Aerial view of Crestuma-Lever dam (https://www.douro.com.pt/blog/rio-douro/a-barragem-de-crestuma-lever) and sampling site (U – Upstream; D – Downstream).

2.1.2 Lake Vela

Lake Vela (40º16'23.743'' N, -8º47'35.149'' W) is located in Figueira da Foz municipality (Coimbra district), approximately 6 km from the sea. Although being the largest lake of a large system of interconnected reservoirs on the surrounding area, is a relatively small (maximum floodable area 70 ha), and shallow (0.9 m average depth; 2.4 m maximum depth) water mass (Antunes et al., 2003; Castro et al., 2005) (Figure 2), and it is classified as eutrophic since 1960 (Nauwerck, 1960). Several studies already demonstrated that this lake shows high levels of nitrates and phosphates, characteristics of eutrophic ecosystems, mainly due to the regular nutrient inputs from intense agricultural and livestock activities in the adjacent areas (Abrantes et al., 2006; Figueiredo et al., 2006; Castro & Gonçalves, 2007). Lake Vela is also characterized by an high turbidity water, without a spring clear water phase, and algal blooms are frequently observed in the warmer seasons (Abrantes et al., 2009).

Fig. 2 - Lake Vela (http://portugalfotografiaaerea.blogspot.pt/2017/04/lagoa-da-vela.htm) and sampling site.

2.1.3 Test Organisms

For this work, two cladocerans species (*Daphnia magna* and *Daphnia longispina*) were selected due to their importance on the zooplanktonic composition in lentic freshwater ecosystems. Cladocera organisms are known for their high sensibility to stressors, high fertility rates, short life cycles and low genetic variability since they adopt an asexual reproduction, by parthenogenesis, under normal environmental conditions. All these characteristics made cladocerans of major interest for the scientific community and used as standard laboratory organisms. *Daphnia* spp. are cladocerans commonly found in lentic freshwater ecosystems and frequently used in ecotoxicological studies. *Daphnia* spp. is a filter freshwater microcrustacean with diversified food sources (bacteria, microalgae), with a small size and a very characteristic morphology (Figure 3). *Daphnia magna* (*D. magna*) is a model organism widely used in aquatic ecotoxicology and ecology studies (Ghazy et al., 2009; Bergman et al., 2011; Lari et al., 2017) and therefore it was selected to carry out this study. However, *D.magna* is not a Portuguese autochthonous species. *Daphnia longispina* (*D.longispina*) was a field species chosen for this study since it is an autochthonous species from Portuguese lentic freshwater ecosystems. Although this species presents smaller dimensions than *D. magna*, it has similar laboratory requirements.

D. magna and *D. longispina* cultures were fed three times a week when the culture medium was renewed and reared under a16h^L:8h^D photoperiod and a temperature of 20±2 ºC on a climatic chamber (Incubator TC 445 S, Lovibond® Water Testing). Neonates born between the 3rd to 5th broods were used for renewing the culture or for initiating assays.

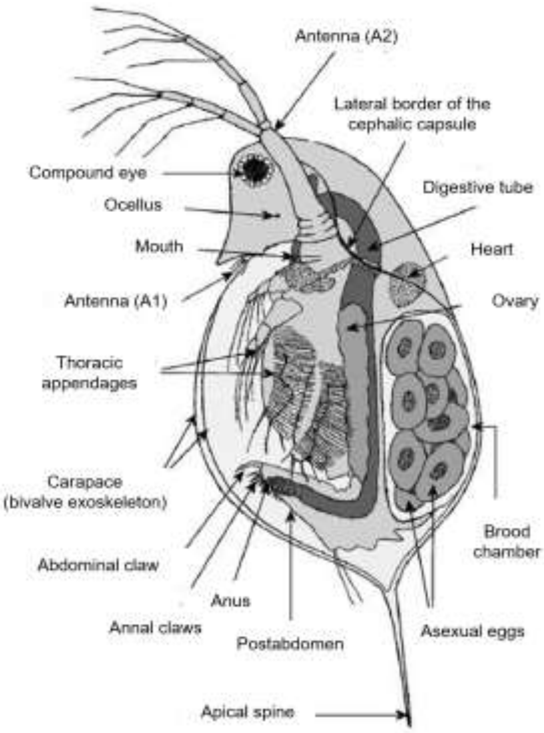

Fig. 3 - *Daphnia* illustration adapted from Antunes and Castro (2017).

2.2 Sampling Procedures

The sampling of *D. longispina* for this work took place in the early spring at Crestuma reservoir and lake Vela. *D. longispina* organisms were collected, to conduct individual laboratory cultures, using a plankton net with a 150 µm mesh. Samples were stored in plastic bottles and transported to the laboratory for taxa identification and initiate individual cultures.

Additionally, water samples were collected at each site, in two seasons (*summer – worst case scenario; and spring – best case scenario*) in order to characterize the water quality and conduct the laboratory tests. Water samples (5 L) were collected to perform the tests and for further chemical analysis (nitrates and total phosphorous). Water temperature (ºC), conductivity (µS/cm), dissolved oxygen (mg/L and %) and pH were determined *in situ* using a multi-parameter probe (WTW Multi 350i/SET).

2.3 Laboratory Procedures

2.3.1 Water physical and chemical analysis

Water samples were processed according to the physical and chemical parameters established by the Water Frame Directive (WFD) for artificial and heavily modified surface water bodies. To evaluate total phosphorus and nitrates, the water was primarily mineralized with potassium peroxodisulfate ($K_2S_2O_8$). Total phosphorus was determined using the methodology described by APHA (1989). In this method, the mineralized water samples react with ammonium molybdate ($(NH_4)2MoO_4$) and are reduced by tin chloride and gain a blue color. Samples were read on the spectrophotometer at 690 nm and total phosphorus was quantified according to a standard calibration curve. Nitrates were determined by an adaptation of the cadmium reduction method. A photometric test was performed using a Spectroquant Multy Colorimeter according to standard procedures.

2.3.2 *Daphnia* spp. culture maintenance

At the laboratory, cladoceran *taxa* were identified with specific keys (Amoros, 1984) through a binocular stereoscope and *D. longispina* isolated for individual cultures.

Monoclonal *D. magna* and *D. longispina* populations were maintained for several generations under laboratory standard conditions. For culture maintenance, a synthetic hard water medium "ASTM hard water" was used (ASTM, 1980). The medium was supplemented with a standard organic additive (*Ascophyllum nodosum* extract) since ASTM is a poor nutrient medium (Baird et al., 1988).

The organisms were fed with *Raphidocelis subcapitata* (freshwater microalgae formerly known as *Pseudokirchneriella subcapitata*) with a ratio of 3.0×10^5 cells.mL^{-1}.day^{-1}. *D. longispina* is smaller when compared to *D. magna* and therefore *R. subcapitata* was added with a ratio of 1.5×10^5 cells.mL^{-1}.day^{-1}. Microalgae cultures were kept in Woods Hole MBL medium (Stein, 1973) under controlled conditions of temperature (20±2 ºC) and photoperiod (16h^L:8h^D). After 7 days growing, cultures reached exponential phase (OECD, 2006) and were harvested. Cultures were then centrifugated at 3900 rpm (rotations per minute) for 5 minutes. The supernatant was discarded and the pellet resuspended in ASTM. The obtained suspension was diluted in a 1:10 proportion and the absorbance was measured at λ=440 nm on a spectrophotometer. A standard volume of food was added to the cultures depending on algae cell concentrations, which was calculated based on the correlation of the absorbance measured values (maintained between 0.400 and 0.900).

2.3.3 Experimental Procedure: Feeding Inhibition Tests

The feeding inhibition tests were performed according to the methodology described by McWilliam and Baird (2002). Two different assays were design based on the two aims of this study. *D. magna* and *D. longispina* (from Crestuma reservoir and lake Vela) were used as test organisms in both assays. All treatments had 5 replicates, with 5 organisms each, and a blank control to account for the potential algae growth during the test period. Neonates 4 or 5 days old born between the 3rd to 5th broods were used for all the tests.

The treatments volume used on both assays was 100 mL of the medium (or natural water) and *R. subcapitata* was added to the vessels with a ratio of 3.0 x 10^5 cells.mL^{-1}.day^{1} for *D. magna* and 1.5 x 10^5 cells.mL^{-1}.day^{-1} for *D. longispina*. The vessels absorbance was then measured at λ=440 nm ($AbsFI_0$) with a spectrophotometer and after this measurement, 5 organisms were added to each vessel. Afterward, the vessels were placed in a climate chamber for 24 h at 20 ºC, and in total darkness to avoid algae growth. After the assay period, each vessel absorbances was once again measured and registered ($AbsFI_{24}$). Feeding rate was calculated according to the following equation:

$$F = ((V * (AbsFI_0 - AbsFI_{24})) / t) / n$$

where V corresponds to the assay volume used (100 mL), t stands for the assay period (24 h) and n to the number of organisms per vessel/replicate (n=5) (Allen et al., 1995).

2.3.3.1 Seston quality assay

In order to evaluate the seston quality in a natural worst-case and best-case scenario, the water sampling occurred at two distinct seasons, at the end of the summer and beginning of spring. In the summer, the water quality is worse as a consequence, for example, of the increase of effluents discharge or increase of recreational activities. Therefore, summer season was considered the worst-case scenario. On the contrary, spring season was considered the best-case scenario in this study.

To perform the feeding inhibition tests to assess the seston nutritional quality of Crestuma reservoir and lake Vela filtered and unfiltered water, from each site, were used as treatments. These two treatments allowed to evaluate the effect of the seston presence in the feeding rate of the organisms. For the filtered treatment, water samples were filtrated through a glass microfiber filter with a 1.2 μm porosity, 47 mm diameter (Whatman GF/C filter), using a vacuum pump. The unfiltered treatment was the water samples used directly without filtrations or another handling. ASTM was used as control treatment.

2.3.3.2 Salinity assay

Nonlethal concentrations of sodium chloride (NaCl) were used to evaluate the effect of increasing salinity on the food performance of *Daphnia* spp.. The concentrations tests on *D. magna* were selected based on Gonçalves et al. (2007) and Martínez-Jerónimo and Martínez-Jerónimo (2007) studies (EC_{50}/LC_{50}) and ranged from 0.7 g/L to 3.3 g/L of NaCl, using a dilution factor of 1.35. For *D. longispina* the tested concentrations were selected according to values already tested by Gonçalves et al. (2007) (EC_{50}), Leitão et al., (2013) (LC_{50}) and Loureiro et al., (2015) and ranged from 0.7 g/L to 1.0 g/L of NaCl, using a dilution factor of 1.1. ASTM was used as control treatment.

2.4 Statistical Analysis

A one-way ANOVA was done to test the differences between the treatments of each feeding test (seston quality and salinity). Previously, data were tested for normality by the Shapiro-Wilk test and for homogeneity of variances by the Levene's test, since data normality and homogeneity are conditions for the one-way ANOVA application. When the ANOVAs were statistically significant ($p<0.05$), a Dunnett's test was applied to discriminate which concentrations were significantly different from the control group. In the salinity assay, this procedure allowed the determination of the standard no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values. All the statistical analysis was done using the SPSS 25 software package for Windows (IBM® SPSS® Statistics, New York, USA).

3. Results

3.1 Aquatic ecosystem characterization: physical and chemical parameters

The water quality parameters used to classify heavily modified and artificial superficial water bodies can be applied to the category of natural surface water which most closely resembles the water body in question (INAG, 2009). Therefore, for lakes and lagoons, reservoirs are the most closely resemble water body. Table 1 presents the range of values for physical and chemical parameters proposed in Water Framework Directive (WFD) obtained in the two sampling periods, for the two lentic ecosystems studied, and the respective comparison to the maximum thresholds values established for the "Good Ecological Potential" (GEP) for northern heavily modified and artificial water bodies.

Table 1 - Comparison between the established limits for physical and chemical parameters for Good Ecological Potential in northern reservoirs established by WFD and the results obtained in the sampling sites and periods. Bold values stand for values outside the established thresholds.

Parameters	Northern reservoirs	Crestuma reservoir		Lake Vela	
		Summer	Spring	Summer	Spring
Dissolved oxygen (mg/L)	≥5	6.29	**3.20**	**1.35**	**2.02**
Oxygen saturation rate (%)	60 – 120	77.9	**35.1**	**19.0**	**22.5**
pH	6 – 9	7.48	**5.48**	7.47	**5.68**
Nitrates (mg/L)	≤25	3.0	1.4	2.1	0.8
Total phosphorus (mg/L)	≤0,05	0.04	0.03	**385.34**	**0.20**
Temperature (ºC)	-	24.8	19.2	21.7	20.5
Conductivity (μS/cm)	-	276	211	700	473

Dissolved oxygen (mg/L and %) show values below the minimum of 5 mg/L, with exception of the water sample from Crestuma reservoir in summer. The two sampling sites showed neutral pH values at the end of summer and acidic pH values in spring, the latter below the range of 6 – 7 required for the classification of Good Ecological Potential. Nitrates concentrations showed for all the samples, from the two sites, values below the maximum established (25 mg/L) for a good ecological potential classification. For the concentration of the total phosphorous analysis, no seasonal variations at Crestuma reservoir were recorded and the values remained below of the maximum limit required for the Good Ecological Potential classification. However, for lake Vela, these values were always above the 0.05 mg/L limit.

Overall, regarding the analyzed physical and chemical water parameters, only summer water samples from Crestuma reservoir were within the considered thresholds for the good ecological potential classification. Contrarily, considering the majority of these parameters, neither summer nor spring's lake Vela water samples were within the limits.

3.2 Feeding Inhibition Tests

3.2.1 Seston quality assay

Figures 4 and 5 show the results obtained on the feeding inhibition tests performed with the natural waters sampled from Crestuma reservoir and lake Vela (end of summer (2017) and the beginning of spring (2018)), with *D. magna*. Regarding the results obtained with the water collected in summer, a significant decrease in the feeding rate was observed between the control treatment and the unfiltered and water from Crestuma treatments filtered ($F_{[2, 11]}$=10.956; p=0.004). While with water from lake Vela, a significant decrease in the feeding rate of *D. magna* was only observed on the filtered water treatment ($F_{[2, 13]}$=22.448; p<0.001) (Figure 4).

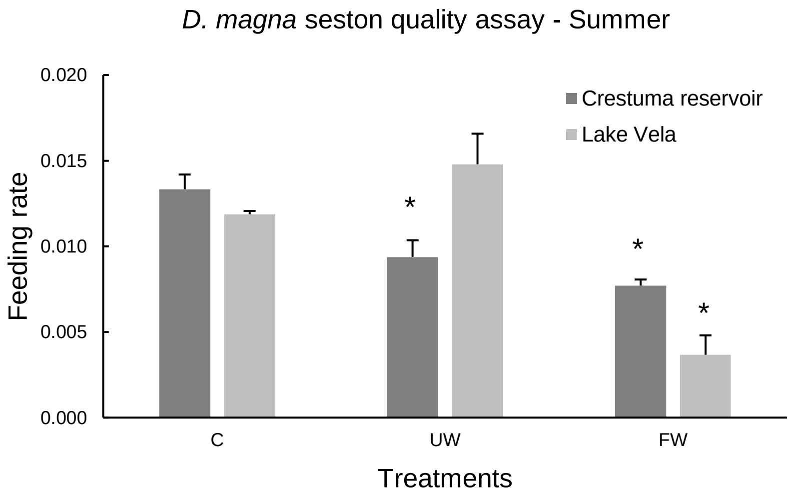

Fig. 4 - Variation of feeding inhibition rate of *D. magna* when exposed to natural waters from the two sampling sites (Crestuma reservoir and Lake Vela), compared to the control treatment (C – Control treatment; UW – Unfiltered water treatment; FW – Filtered water treatment). Data from the end of summer is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test.

For the assay with water collected in spring, in the filtered water treatment from Crestuma reservoir, a significant feeding inhibition has occurred ($F_{[2, 13]}$=23.636; p<0.001) (Figure 5). On the other hand, a significant increase in the feeding rate of *D.*

magna when exposed to the unfiltered water from lake Vela was recorded ($F_{[2, 11]}$=22.246; p<0.001) (Figure 5).

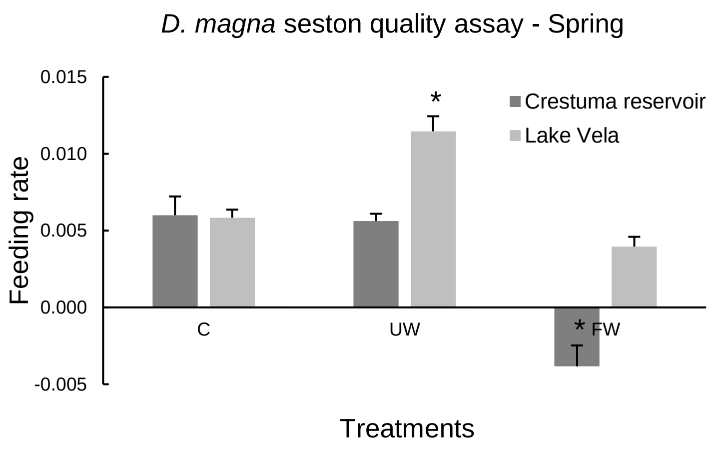

Fig. 5 - Variation of feeding inhibition rate of *D. magna* when exposed to natural waters from the two sampling sites (Crestuma reservoir and Lake Vela), compared to the control treatment (C – Control treatment; UW – Unfiltered water treatment; FW – Filtered water treatment). Data from the beginning of spring is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test.

Figures 6 and 7 present the results of the feeding inhibition tests performed with the natural waters (from Crestuma reservoir and lake Vela) with *D. longispina* isolated from Crestuma reservoir (*D. longispina* C). In the summer season, a significant decrease in the feeding rate of *D. longispina* C after exposed to filtered water from Crestuma was registered ($F_{[2, 13]}$=4.420; p=0.039) (Figure 6). When comparing the treatments with filtered and unfiltered water from lake Vela with the control treatment, significant differences are present ($F_{[2, 13]}$=62.343; p<0.001) (Figure 6). In the treatment with unfiltered water from this site, an increase in the feeding rate occurred. However, when *D. longispina* C was exposed to the treatment with filtered water from lake Vela, a significant feeding inhibition was observed.

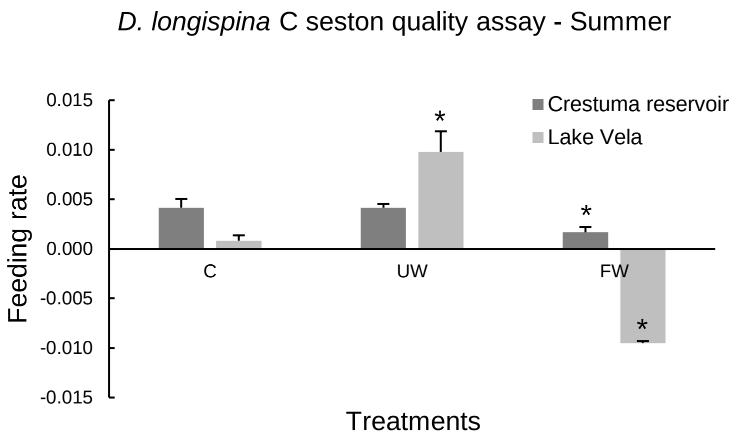

Fig. 6 - Variation of feeding inhibition rate of *D. longispina* C when exposed to natural waters from the two sampling sites (Crestuma reservoir and Lake Vela), compared to the control treatment (C – Control treatment; UW – Unfiltered water treatment; FW – Filtered water treatment). Data from the end of summer is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test.

In the assay performed with water collected in spring, *D. longispina* C was significantly affected between the Crestuma treatment groups ($F_{[2, 13]}$=253.484; p<0.001) and between the Vela treatment groups ($F_{[2, 13]}$=74.745; p<0.001). The feeding rate of *D. longispina* C was significantly inhibited when organisms were exposed to the natural water independently to the treatment (Figure 7). An exception was recorded in the treatment with unfiltered water from lake Vela when a significant increase in the feeding rate was verified.

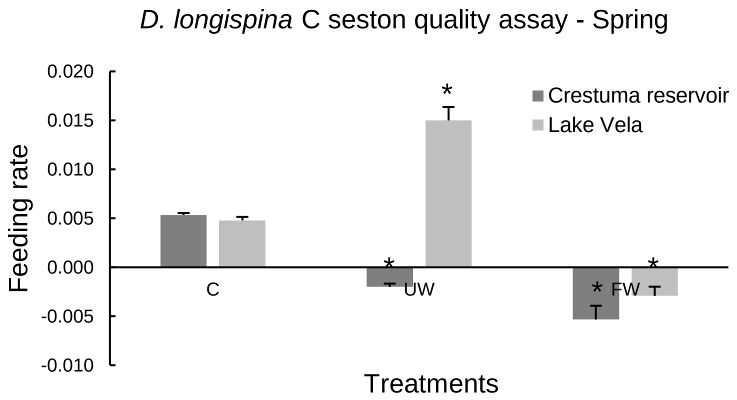

Fig. 7 - Variation of feeding inhibition rate of *D. longispina* C when exposed to natural waters from the two sampling sites (Crestuma reservoir and Lake Vela), compared to the control treatment (C – Control treatment; UW – Unfiltered water treatment; FW – Filtered water treatment). Data from the beginning of spring is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test.

Regarding figures 8 and 9 that presented the results obtained in the feeding inhibition assays with *D. longispina* isolated from the lake Vela (*D. longispina* V), using the natural waters from Crestuma reservoir and lake Vela, from both sampling periods. The assay performed with the summer water samples from Crestuma reservoir did not present significant differences in the feeding rate ($F_{[2, 13]}$=0.136; p=0.874). Contrarily, the assay performed with Vela water samples showed a significant increase of the feeding rate in the *D. longispina* V exposed to the unfiltered water treatment ($F_{[2, 14]}$=13.396; p=0.001) (Figure 8). However, when exposed to the filtered water treatment, *D. longispina* V showed feeding inhibition, although no significant difference was detected.

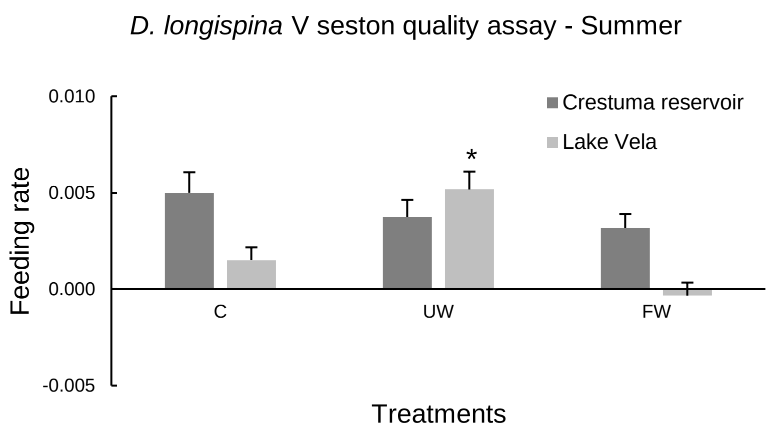

Fig. 8 - Variation of feeding inhibition rate of *D. longispina* V when exposed to natural waters from the two sampling sites (Crestuma reservoir and Lake Vela), compared to the control treatment (C – Control treatment; UW – Unfiltered water treatment; FW – Filtered water treatment). Data from the end of summer is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test.

Regarding the results obtained in the spring assay, on the treatment with unfiltered water from lake Vela a significant increase in the feeding rate of *D. longispina* V was observed ($F_{[2, 11]}$=16.157; p=0.001). On the contrary, when this species was exposed to the filtered water treatment, a significant feeding inhibition was verified. Regarding water from Crestuma reservoir, a significant decrease in the feeding rate of *D. longispina* V when this water was filtered was observed ($F_{[2, 14]}$=15.088; p=0.001) (Figure 9).

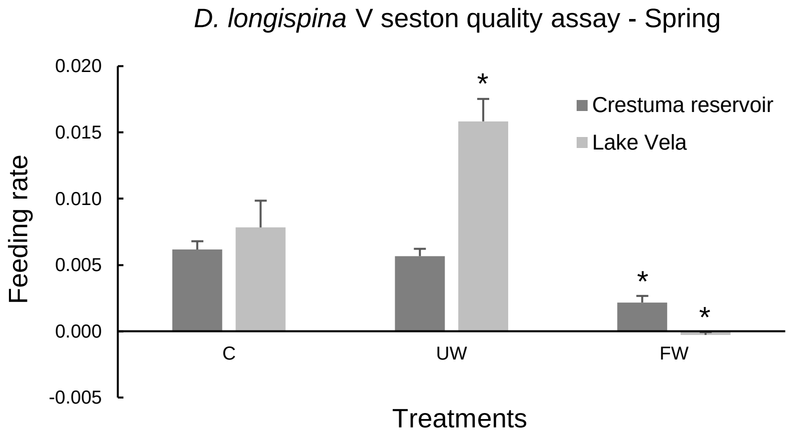

Fig. 9 - Variation of feeding inhibition rate of *D. longispina* V when exposed to natural waters from the two sampling sites (Crestuma reservoir and Lake Vela), compared to the control treatment (C – Control treatment; UW – Unfiltered water treatment; FW – Filtered water treatment). Data from the beginning of spring is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test.

3.2.2 Salinity assay

Figure 10, 11 and 12 present the results of salinity effects in the feeding rate of the *D. magna* and *D. longispina* (C and V). The results showed a significant decrease in the feeding rate for all the species studied: *D. magna* ($F_{[4, 23]}$=11.146; p<0.001), *D. longispina* C ($F_{[4, 23]}$=11.146; p<0.001) and *D. longispina* V ($F_{[4, 21]}$=23.053; p<0.001), along with the NaCl concentration tested. On the assay with *D. magna*, a LOEC was recorded at a concentration of 1.0 g/L of NaCl (Figure 10). Regarding *D. longispina* species, a significant decrease in the feeding rate was observed from 0.8 g/L of NaCl (LOEC). The NOEC value recorded for the 3 populations tested was equal with 0.7 g/L of NaCl. The results here-obtained showed that *D. magna* looks like more tolerant to salinity compared to *D. longispina* species, although both species have been significantly affected by low NaCl concentrations (< 1.0 g/L).

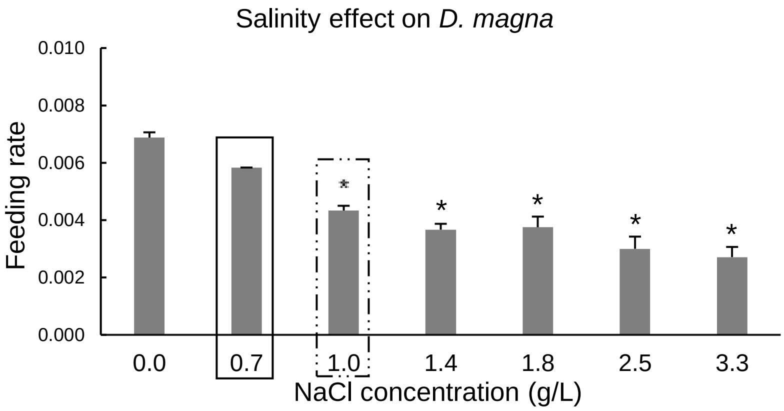

Fig. 10 - Salinity effect on *D. magna.* Data is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test. NOEC —— value; –··–·· LOEC value

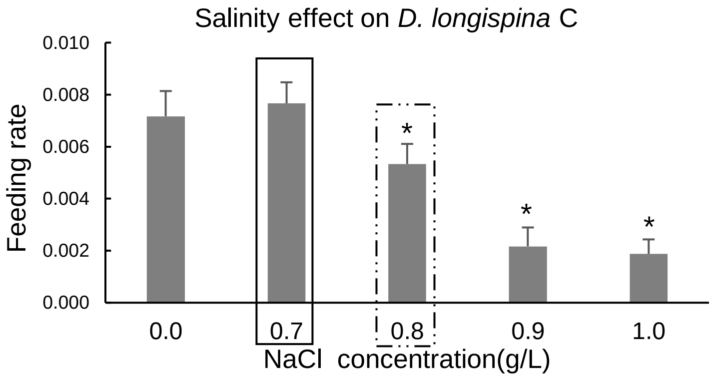

Fig. 11 - Salinity effect on *D.longispina* C. Data is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test. —— NOEC value; –··–·· LOEC value

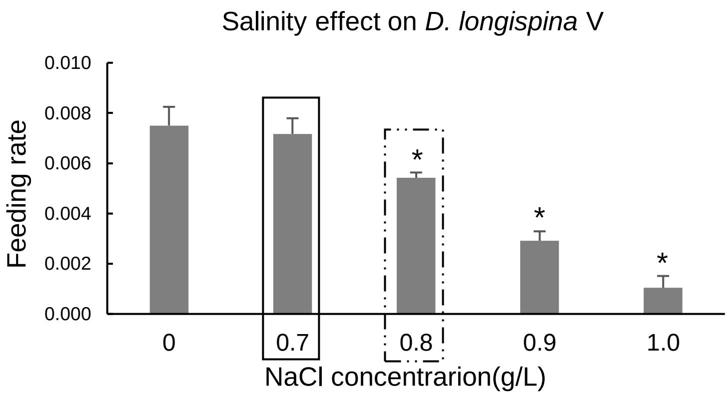

Fig. 12 - Salinity effect on *D.longispina* V. Data is presented as Mean+SEM. *stands for significant differences when compared to control treatment, using the Dunnett's test. —— NOEC value; –··–·· LOEC value

4. Discussion

Environmental changes in lentic freshwater ecosystems can have impacts at any level of the trophic web, altering the water quality and, as a consequence, the structure and diversity of the planktonic community. The nutritional quality and quantity of the seston are essential for the growth and reproduction of zooplankton organisms (Boersma et al., 2001). Moreover, abiotic stresses, as salinity increase, can play an important role in the communities organization (Van Meter et al., 2011). The results here presented showed that *Daphnia* spp. food performance was affected by the seston quality from the water collected in both sites analyzed, as well the salinity concentration.

According to the last data available on *Sistema Nacional de Informação de Recursos Hídricos* (SNIRH), from 2013, the water of Crestuma reservoir had a reasonable quality, which is confirmed by other authors through water quality index determination, physical and chemical water analysis and feeding inhibition tests, for example (Bordalo et al., 2006; da Silva, 2013). The sampling procedures to conduct this study occurred during an atypical summer, described as extremely hot and dry(annual average air temperature 1.1 ºC higher than normal value, being the second hottest year since 1931) (IPMA, 2017), and a cold and rainy spring (IPMA, 2018). The relation between temperature and dissolved oxygen (DO) in aquatic ecosystems is well known, with high temperatures associated with low DO levels and low temperatures with high DO levels (Odum, 1996). However, this relation was not verified in this study, the lowest DO (3.20 mg/L) value occurred when the lower values of temperature were recorded, in the spring season. This situation could be explained by an effluents discharge, rich in organic matter and lixiviation from heavy rains. On the other hand, high values of organic matter cause a decrease in the DO levels. Concerning the water samples from lake Vela, on both seasons were verified low and similar DO levels (mg/L and %), however high turbidity was observed. Namely, summer sampling took place after a massive fire in the area, which could explain the results obtained at this season since the leaching of the ashes causes an alteration in the nutrients dynamic and water turbidity, as consequence, in the DO levels. Additionally, lake Vela has been classified as a eutrophic lake (Nauwerck, 1960; Castro et al., 2005; Abrantes et al., 2009) and one of the most serious effects of eutrophication is DO depletion (Foley et al., 2012). In order to study these hypotheses, other parameters should be addressed in future research such as chemical oxygen demand, biochemical oxygen demand and total nitrogen, although these do not have thresholds defined by the WFD for the GEP classification in heavily modified water bodies.

Nitrates and phosphates are important parameters for the water quality assessment since they control primary production and species composition, whereas the phosphorous is considered the most limiting nutrient in freshwater ecosystems (Xu et al., 2010). There are several sources of N and P inputs to freshwater ecosystems, the majority related to human activities such as wastewater effluents, runoffs from agriculture and pastures (Smith et al., 1999). These nutrients are considered the principals responsible for eutrophication process (Paerl, 2009). Therefore, both parameters are important indicators of pollution and trophic state of freshwater ecosystems. In the water samples collected in Crestuma reservoir, nitrates analysis showed a substantial decrease in the last decade. In 2007 and 2011, according to SNIRH, the annual average for this parameter was 5.6 mg NO_3/L and 5.9 mg NO_3/L, respectively. In a study carried out in 2013, the annual average was 0.034 mg NO_3/L (da Silva, 2013) and, in this work, 3.0 mg NO_3/L (summer) and 1.4 mg NO_3/L (spring) were the values measured. These results are indicative that Crestuma reservoir is not susceptible to contamination by leaching, which is reinforced by the results of the total phosphorous recorded. For the total phosphorous parameter, Crestuma reservoir showed similar values in the two sampling seasons, 0.04 mg P/L in the summer and 0.03 mg P/L in the spring. These values are within the class 2 defined by Nisbet and Verneaux (1970), classifying these waters with low productivity levels. Furthermore, phosphorous concentration appears to be relatively homogeneous along the last years. In this study, the values were slightly above the registered by SNIRH, in which the annual average in 2010 was 0.025 mg P/L. Lake Vela samples also presented low values of nitrates, below the limit established for the Good Ecological Potential (GEP – See table 1). Samples from summer presented values similar to the reported by Abrantes et al. (2006). In the spring season, the nitrates concentration was also below the limit for GEP, contrary to the results obtained in the referred study. Regarding lake Vela results for total phosphorous, both summer and spring samples were much above the maximum limit required for the GEP. As previously referred, summer sampling took place after an intensive fire, which can partly explain the extremely high value obtained, 385.34 mg P/L, typical of pollutant waters (class 6), according to Nisbet and Verneaux (1970). Moreover, lake Vela is surrounded by agricultural fields and, as consequence, inputs of nutrients by leaching are expected and already described (Antunes et al., 2003; Abrantes et al., 2008, 2010). In the spring season sampling, this parameter value suffered a significant reduction, from 385.34 mg P/L to 0.2 mg P/L, however still above of the limit maximum required for the GEP classification. With this result, lake Vela is considered be within the class 4 defined by Nisbet and Verneaux (1970) and therefore, a eutrophic lake with high productivity.

Conductivity was another parameter analyzed *in situ* and measures the quantity of dissolved ions in the water. In the Crestuma reservoir, the results for this parameter were similar between seasons and to the ones obtained by da Silva (2013) (annual average of 210 μS/cm). Furthermore, the results of this study were very similar to the annual average registered in 2011 by SNIRH, which was 251 μS/cm. On lake Vela, this parameter was very high in both sampling season, which could be related to ashes from the fire, in the case of summer.

The analysis of the physical and chemical parameters under the perspective of the WFD indicates that the two lentic freshwater ecosystems studied suffered some variations along the seasons. Both ecosystems showed similar results on the water quality parameters although being under different anthropic pressures. Taking into account these parameters, with the exception of the summer water samples, from Crestuma reservoir, the two types of water (Crestuma and Vela) were of poor quality, regardless of the season. Biological parameters should be associated with the physical and chemical parameters to evaluate the water quality since these two parameters can provide distinct information (Martinez-Haro et al., 2015).

In order to assess the seston quality and functioning of Crestuma reservoir and lake Vela, feeding inhibition tests were performed with *D. magna* and *D. longispina*, collected from both sites (*D. longispina* C a *D. longispina* V). The results of the seston quality assay here presented are indicative that seston from lake Vela has more quality than the seston from Crestuma reservoir, regardless the results of the physical and chemical parameters observed. When *Daphnia* spp. were exposed to the summer water samples from Crestuma, it was verified that the seston had nutritional quality only to *D. longispina* spp. *(D. longispina* C and D. longispina V). On the other hand, when daphnids were exposed to the spring water samples from Crestuma, the results suggest that the seston quality was insufficient for *D. longispina* C since it was the only species with a significant feeding inhibition in the unfiltered water. This water samples showed a poor quality, taking into account the parameters proposed by the WFD. Therefore, the feeding rate of *D. longispina* C, a sensitive species, decreased, reinforced the hypothesis that feeding behavior is affected by the water quality. *D. magna* is considered a more tolerant species than *D. longispina*, which could explain not being affected by the poor quality of Crestuma water and seston in this season. On the contrary, the results presented by da Silva (2013) showed a significant increase in the feeding rate of *D. magna* in this season, however, the water quality in this period was significantly better. As for *D. longispina* from lake Vela, this population is adapted to a eutrophic ecosystem that shows poor water quality and, as consequence, it is expected to be more tolerant to alterations in the water quality.

Concerning the assays with the summer and spring water samples from lake Vela, although the results observed in the physical and chemical water parameters, the seston showed nutritional quality for all *Daphnia* spp. tested. It was verified a significant increase in the feeding rate of these species in the unfiltered water treatment in the majority of the assays, independently of the season.

It is known that phytoplankton species show different nutritional quality (Ahlgren et al., 1990; von Ruckert & Giani, 2008) and therefore, not only the quantity but also composition of the phytoplanktonic community is essential to the zooplanktonic community (Abrantes et al., 2006). Taking this into account, it is possible that the phytoplanktonic community of lake Vela is more diverse and abundant than the phytoplanktonic community of Crestuma reservoir. Moreover, these results can be related to cyanobacteria presence in Crestuma water since these cannot only inhibit the filter capacity of some zooplankton species but also release toxic metabolites that remain in the water, even after filtration (Müller-Navarra & Lampert, 1996; Jang et al., 2003; Freitas et al., 2014; Ger et al., 2014). In this scenario, the treatment with filtered water will cause feeding inhibition since the organisms are at the same way exposed to toxins, and on the other hand, do not have sufficient food available to cope. Further research should include the analysis of the phytoplanktonic community, as proposed by WFD, in order to confirm this approach. Moreover, a more efficient filtration method, capable of remove possible toxins of the water, should be tested, such as ultrafiltration or nanofiltration (Gijsbertsen-Abrahamse et al., 2006; Merel et al., 2013).

The assay performed to evaluate the effect of salinity on daphnis showed that *D. magna* and *D. longispina* (C and V) were affected by low NaCl concentrations. In this work, the results of the feeding rate parameter showed a LOEC value for *D. magna* of 1.0 g/L (NaCl) and a NOEC of 0.7 g/L (NaCl). Gonçalves et al. (2007) reported a LOEC value of 5.0 g/L and a NOEC of 4.55 g/L when analyzing survival and life history parameters under salinity stress. In another study, the results showed that *D. magna* can grow under concentrations up to 6.0 g/L NaCl (Martínez-Jerónimo & Martínez-Jerónimo, 2007). These results showed that the feeding rate is affected at lower concentrations than the growth and survival, reinforcing that this species can not survive with high salinities (Arnér & Koivisto, 1993; Ghazy et al., 2009). For *D. longispina*, the same trend is observed, and the here presented results showed a LOEC of 0.8 g/L and a NOEC of 0.7 g/L of NaCl. A LOEC value of 2.07 g/L and a NOEC value of 1.88 g/L of NaCl were reported in another study for the majority of life history parameters for *D. longispina* (Gonçalves et al., 2007). The LOEC value of the two species studied indicates that *D. longispina* is more sensitive than *D. magna*, which is confirmed by other authors on different studies (Koivisto, 1995; Gonçalves et al., 2007). This different sensibility of *D.*

magna and *D. longispina* was already observed by Bossuyt and Janssen (2005) when exposing different cladoceran species, including *D. magna* and *D. longispina*, to copper. Moreover, the authors verified that *D. magna* is less sensitive to this abiotic stress. Muyssen et al. (2005) observed the same tendency when studying cladoceran populations tolerance to zinc. Regarding the here presented results, however, the difference between the referred LOEC values is minimal and there is no difference between the NOEC values obtained. Therefore, although being different species, this study showed that the salinity stress has a similar effect on them.

5. Conclusion

The results obtained in this study indicate that the feeding inhibition tests should be used in association with other parameters when evaluating the water quality of lentic freshwater ecosystems. Overall, it was possible to verify that the seston from lake Vela had more nutritional quality than the seston from Crestuma reservoir, for *Daphnia* spp. However, in this study an environmental disturbance that was not expected was introduced, the fire occurred in the lake Vela area. This fire was an extreme event and correspond to a worst-case scenario with significant impacts in aquatic ecosystems. Further research should include methodology capable of acknowledging these type of disturbances.

Regarding the salinization impact, this study reinforces results obtained in previous works, showing that *Daphnia* spp. is affected even by lower NaCl concentrations. Furthermore, it was verified that feeding inhibition tests can be used as a tool to evaluate the impact of abiotic stresses such as salinity, being a cost-effective tool.

6. References

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